Method

Field of the Invention

This invention relates to methods of modulating the proteolytic processing of amyloid precursor protein (APP) and methods of treatment of conditions in which abnormal processing of amyloid precursor protein (APP) is implicated such as Alzheimer's disease.

Background to the Invention

Alzheimer's disease (AD) is characterised by the presence of two diagnostic features of pathology. These are amyloid plaques and neurofibrillary tangles composed of aggregated beta-amyloid peptide (Aβ40 and Aβ42) and hyperphosphorylated tau respectively (Dawbarn & Allen 2001 Neurobiology of Alzheimer's Disease OUP).

A comprehensive study has shown a strong link in patients between beta-amyloid accumulation and cognitive decline (Naslund et al 2000). This is consistent with genetic and epidemiological studies that suggest that some mutations in APP and presenilin genes can predispose to early onset AD, which mutations also enhance the levels of Aβ40 and Aβ42 peptide, including the ratio thereof.

Cleavage of the type I transmembrane amyloid precursor protein (APP) by two distinct proteases designated beta- and gamma-secretase is necessary for the formation of beta-amyloid peptide. The molecular identity of beta-secretase as the aspartyl-protease Asp2/BACE1 has been confirmed (Hussain et al 1999; Vassar et al 1999). The nature of gamma-secretase remains the source of some debate and is likely to consist of a high molecular weight complex consisting of at least the following proteins: presenilins, Aph1, Pen2 and nicastrin (reviewed in Medina & Dotti Cell Signalling 2003 15(9):829-41).

The processing of APP within the CNS is likely to occur within a number of cell-types including neurons, oligodendrocytes, astrocytes and microglia. While the overall rate of APP processing in these cells will be influenced by the relative level of expression of APP, BACE1/Asp2, presenilin-1 and -2, Aph1, Pen2 and nicastrin.

Furthermore, additional factors regulating the subcellular location of APP can also influence its processing as shown by the finding that mutation of the YENP motif in the APP cytoplasmic domain which blocks its endocytosis reduces beta-amyloid production (Perez et al 1999 J Biol Chem 274 (27) 18851-6). Retention of the APP-beta-CTF in the ER by the addition of the KKQN retention motif is sufficient to reduce amyloid production in transfected cells (Maltese et al 2001 J Biol Chem 276 (23) 20267-20279). Conversely, elevation of endocytosis, by overexpression of Rab5 is sufficient to elevate amyloid secretion from transfected cells (Grbovic et al 2003 J Biol Chem 278 (33) 31261-31268).

Consistent with these findings further studies have shown that reduction of cellular cholesterol levels (a well known risk factor for AD) reduced beta-amyloid formation. This change was dependent on altered endocytosis as demonstrated by the use of the dominant negative dynamin mutants (K44A) and overexpression of the Rab5 GTPase activating protein RN-Tre (Ehehalt et al 2003 J Cell Biol 160 (1) 113-123).

Cholesterol rich microdomains or rafts are also an important cellular site of beta-amyloid production and APP, BACE1 and components of the gamma-secretase complex have all been shown to transiently reside within rafts. Antibody cross-linking of APP and BACE1 towards cholesterol rich rafts was able to elevate beta-amyloid production (Ehehalt et al 2003 J Cell Biol 160 (1) 113-123). Expression of GPI-anchored BACE1, which is exclusively targeted to lipid rafts, is similarly able to elevate APP cleavage and beta-amyloid production (Cordy et al 2003 PNAS 100(20) 11735-11740).

Neuronal growth is finely balanced by the activities of positive factors such as neurotrophins like NGF and matrix proteins like laminin and the opposing activities of inhibitory factors such as ephrins, MAG, semaphorin-3A and NogoA (reviewed in Tessier-Lavigne & Goodman 2000 Science 287(5454):813-4). The antagonistic nature of the signalling from these factors is illustrated by the finding that enhanced neuronal growth correlates with a reduction in RhoA activation (Nusser et al 2002 J Biol Chem 277 (39) 35840) and the inhibition of neuronal growth results in increased RhoA activation (Niederost et al 2002 J Neuroscience 22(23) 10368-10376).

Nogo is a protein found in the myelin sheath that has inhibitory action on axonal growth (Prinjha, R et al (2000) Nature 403, 383-384; GrandPre, T et al (2000) Nature 403, 439-444 and Chen, MS et al (2000) Nature 403, 434-439).

At least three Nogo isoforms are generated by alternative splicing of transcripts derived form the NogoA gene. The C-terminal third of all three isoforms shares high homology (approximately 70% at the amino acid level) with the reticulon protein family. NogoA, the largest isoform, has been shown to inhibit axon regeneration in culture. It is thought that the normal role of Nogo proteins is to prevent axon sprouting in the uninjured central nervous system. NogoA is localised to central nervous system myelin and is highly expressed in oligodendrocytes; NogoB and NogoC are expressed in some neurons and several non-neural tissues. All Nogo isoforms surprisingly have a C-terminal ER-retention motif but at least some of NogoA protein is thought to reach the cell surface. All 3 Nogo isoforms have 2 potential trans-membrane domains. Both the C and N termini may be cytoplasmically exposed and a 66 amino acid loop separated by the TM domains may be located extracellularly.

It has been reported that BACE and Nogo may associate in a physical sense, but such reports are based solely on immunoprecipitation experiments (for example WO02/058323 and WO03/088926). There has been no further confirmation of this association and no experimental evidence of any functional interaction between BACE and Nogo.

WO03/088926 discloses that cells expressing RTN3, a member of the reticulon family, have altered levels of Aβ release compared with negative controls. However there is no indication as to whether this alteration is an increase or decrease in Aβ levels and hence whether the search for an antagonist or an agonist of RTN3 would be a fruitful avenue of research. He et al (Nature Medicine 2004 10(9) 959-965) subsequently suggested that elevation of Reticulon 3C expression by transfection into cells overexpressing human APP with swedish mutations could reduce production of Aβ1-40. No data was presented on effects on Aβ1-42 and no measures of culture viability were presented to exclude non-specific effects. In direct contrast to the present invention these studies indicated that it would be necessary to identify an agonist

of reticulons to inhibit amyloid generation. Furthermore, this study did not look at the ability of NogoA (Reticulon 4A) to modulate amyloid peptide formation.

WO04/093893 discloses experimental evidence suggesting that APP (and Aβ peptide) interact with the Nogo receptor. These same experiments failed to show any interaction between APP and NogoA. Further experimental data presented in WO04/093893 shows that treatment of APPsw/PSEN-1(Delta E9) double transgenic mice with sNgR310-Fc, a Nogo receptor antagonist, resulted in a reduction of the level of Aβ deposition into plaques. The data presented suggest that neither NogoA fragments nor NogoA antagonists would have a similar effect on Aβ production as that observed for Nogo receptor polypeptides and Nogo receptor antagonists.

The process of neurodegeneration underlies many neurological diseases/disorders including, but not limited to, acute diseases such as stroke, traumatic brain injury and spinal cord injury as well as chronic diseases including Alzheimer's disease, fronto-temporal dementias (tauopathies), peripheral neuropathy, Parkinson's disease, Huntington's disease, Creutzfeldt-Jakob Disease (CJD), amyotropic lateral sclerosis (ALS), multiple sclerosis and inclusion body myositis.

The identification of therapeutic and prophylactic treatments for neurodegenerative diseases, in particular Alzheimer's disease, remains a highly desirable goal for the pharmaceutical industry and for healthcare providers.

Summary of the Invention

The present invention is based on the finding that antagonists of Nogo influence the production of the amyloidogenic Aβ peptide. This novel finding provides an unexpected route for therapeutic intervention in disorders resulting from abnormal production of Aβ peptide in particular Alzheimer's disease.

Accordingly the invention provides a method of modulating Aβ peptide production using an antagonist of Nogo. Further the invention provides methods of modulating BACE activity, methods of modulating amyloid deposition, pharmaceutical compositions and methods for the treatment of neurodegenerative diseases such as Alzheimer's disease.

Detailed description of the invention

In a preferred aspect the invention provides a method of modulating production of an amyloidogenic peptide comprising contacting a cell which is expressing:

a) the precursor from which the amyloidogenic peptide is derived; and

b) a Nogo polypeptide

with a Nogo antagonist.

In a preferred embodiment the precursor is APP. Preferably the amyloidogenic peptide is Aβ, most preferably Aβ40, Aβ42 or a combination of both.

In a further preferred embodiment the Nogo polypeptide is NogoA.

The Nogo antagonist may be a small organic molecule, peptide, polypeptide or antibody that binds to Nogo and thereby inhibits or extinguishes Nogo activity. In another aspect the Nogo antagonist may be any such molecule other than an antibody. Other possible Nogo antagonists include peptides, polypeptides, oligonucleotides or polynucleotides (for example aptamers) that binds to the same site on the Nogo receptor molecule as the Nogo polypeptide, but without inducing Nogo receptor-induced activities, thereby preventing the action of the Nogo receptor by excluding Nogo from binding.

Further potential antagonists include small molecules which bind to and occupy a binding site on the Nogo polypeptide thereby preventing binding to cellular binding molecules, such as receptor molecules, such that normal Nogo biological activity is prevented or reduced. Examples of such small molecules include, but are not limited to small organic molecules, peptides or peptide-like molecules, polypeptides, oligonucleotides or polynucleotides (for example aptamers).

In still another approach, expression of the gene encoding endogenous Nogo polypeptide can be inhibited using expression blocking techniques. Known such techniques involve the use of antisense sequences, either internally generated or separately administered (see, for example, O'Connor, J Neurochem (1991) 56:560 in Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988)). Alternatively, oligonucleotides which form triple helices ("triplexes") with the gene can be supplied (see, for

example, Lee *et al.*, Nucleic Acids Res (1979) 6:3073; Cooney *et al.*, Science (1988) 241:456; Dervan *et al.*, Science (1991) 251:1360). These oligomers can be administered *per se* or the relevant oligomers can be expressed *in vivo*.

Synthetic antisense or triplex oligonucleotides may comprise modified bases or modified backbones. Examples of the latter include methylphosphonate, phosphorothioate or peptide nucleic acid backbones. Such backbones are incorporated in the antisense or triplex oligonucleotide in order to provide protection from degradation by nucleases and are well known in the art. Antisense and triplex molecules synthesised with these or other modified backbones also form part of the present invention.

In addition, expression of the Nogo polypeptide may be prevented by using ribozymes specific to the Nogo mRNA sequence. Ribozymes are catalytically active RNAs that can be natural or synthetic (see for example Usman, N, et al., Curr. Opin. Struct. Biol (1996) 6(4), 527-33.) Synthetic ribozymes can be designed to specifically cleave Nogo mRNAs at selected positions thereby preventing translation of the Nogo mRNAs into functional polypeptide. Ribozymes may be synthesised with a natural ribose phosphate backbone and natural bases, as normally found in RNA molecules. Alternatively the ribozymes may be synthesised with non-natural backbones to provide protection from ribonuclease degradation, for example, 2'-O-methyl RNA, and may contain modified bases.

Further, expression of the Nogo polypeptide may be prevented using synthetic short interfering (si) double stranded RNA molecules to transiently suppress Nogo gene function. This technique of RNA interference (RNAi), originally coined from work in *C.elegans* (A. Fire, Trends Genet., 1999, **15**(9), 358) was later developed such that its use could be applied to mammalian cells (S. M. Elbashir, J. Harborth, W. Lendeckel, A. Yalcin, K. Weber, T. Tuschl, Nature, 2001, **411**, 494). Once correctly localised the antisense strand of the RNA duplex binds to the complementary region of the targeted messenger (m)RNA (coding for the target of interest), leading to hydrolysis of the mRNA and its subsequent degradation. This transient reduction in mRNA leads to a transient reduction in target gene expression.

Preferably the Nogo antagonist is an antibody, more preferably a monoclonal antibody.

Preferably the monoclonal antibody is a function-blocking anti-Nogo monoclonal antibody, more preferably a function-blocking anti-NogoA monoclonal antibody.

Preferably the function-blocking anti-NogoA monoclonal antibody comprises CDR's as shown in the tables 1 to 6 which show the CDRs of three independently isolated anti-Nogo antibodies: 2A10/3, 2C4/1 and 15C3/3. The CDR's are identified as described by Kabat (Kabat et al. (1991) "Sequences of proteins of immunological interest"; Fifth Edition; US Department of Health and Human Services; NIH publication No 91-3242). CDRs preferably are as defined by Kabat but following the principles of protein structure and folding as defined by Chothia and Lesk, (Chothia et al., (1989) "Conformations of immunoglobulin hypervariable regions"; Nature 342, p877-883) it will be appreciated that additional residues may also be considered to be part of the antigen binding region.

Table 1: Antibody 2A10/3 light chain CDRs

CDR	***According to Kabat***
L1	RSSKSLLYKDGKTYLN (SEQ ID NO:1)
L2	LMSTRAS (SEQ ID NO:2)
L3	QQLVEYPLT (SEQ ID NO:3)

Table 2: Antibody 2A10/3 heavy chain CDRs

CDR	***According to Kabat***
H1	SYWMH (SEQ ID NO:4)
H2	NINPSNGGTNYNEKFKS (SEQ ID NO:5)
H3	GQGY (SEQ ID NO:6)

Table 3: Antibody 2C4/1 light chain CDRs

CDR	*According to Kabat*
L1	RSSQSLVHSNGNTYLH (SEQ ID NO:7)
L2	KVSNRFS (SEQ ID NO:8)
L3	SQSTHVPLT (SEQ ID NO:9)

Table 4: Antibody 2C4/1 heavy chain CDRs

CDR	*According to Kabat*
H1	FSCYAMS (SEQ ID NO:10)
H2	SISDGGSYTYYPDNVKG (SEQ ID NO:11)
H3	ELLFDY (SEQ ID NO:12)

Table 5: Antibody 15C3/3 light chain CDRs

CDR	*According to Kabat*
L1	RSSKSLLHSNGNTYLY (SEQ ID NO:13)
L2	RMSNLAS (SEQ ID NO:14)
L3	MQHLEYPLT (SEQ ID NO:15)

Table 6: Antibody 15C3/3 heavy chain CDRs

CDR	*According to Kabat*
H1	SYWMN (SEQ ID NO:16)
H2	QIYPGDGDTNYNGKFKG (SEQ ID NO:17)
H3	RFDY (SEQ ID NO:18)

The anti-Nogo antibody may also be an antibody which binds to the same epitope on the Nogo polypeptide as an antibody having the CDRs described above. Preferably the epitope comprises the region 586 to 785 (NogoA amino acid numbering). More preferably the epitope is comprised within the region 586 to 685 or 686 to 785. Competitive inhibition assays are used for mapping of the epitopes on an antigen.

The anti-Nogo antibody may be a chimeric antibody which binds to and neutralises Nogo, preferably human Nogo, comprising CDRs such as those disclosed in tables 1 to 6. Preferably the chimeric antibody comprises mouse and human sequences.

Preferably the anti-Nogo antibody is a humanised antibody which binds to and neutralises Nogo, preferably human Nogo.

Preferably the anti-Nogo antibody is an antibody or functional fragment thereof which comprises a heavy chain variable domain which comprises one or more CDR's selected from CDRH1, CDRH2 and CDRH3 of table 2, preferably comprising at least CDRH3, and/or a light chain variable domain which comprises one or more CDRs selected from CDRL1, CDRL2 and CDRL3 from table 1; an antibody or functional fragment thereof which comprises a heavy chain variable domain which comprises one or more CDR's selected from CDRH1, CDRH2 and CDRH3 of table 4, preferably comprising at least CDRH3, and/or a light chain variable domain which comprises one or more CDRs selected from CDRL1, CDRL2 and CDRL3 from table 3; or an antibody or functional fragment thereof which comprises a heavy chain variable domain which comprises one or more CDR's selected from CDRH1, CDRH2 and CDRH3 of table 6, preferably comprising at least CDRH3, and/or a light chain variable domain which comprises one or more CDRs selected from CDRL1, CDRL2 and CDRL3 from table 5.

More preferably the anti-Nogo antibody or functional fragment thereof comprises:

a) a heavy chain variable domain (V_H) which comprises in sequence CDRH1, CDRH2 and CDRH3 from table 2,

and /or

b) a light chain variable domain (V_L) which comprises in sequence CDRL1, CDRL2 and CDRL3 from table 1;

an anti-Nogo antibody or functional fragment thereof which comprises:

a) a heavy chain variable domain (V_H) which comprises in sequence CDRH1, CDRH2 and CDRH3 from table 4,

and /or

b) a light chain variable domain (V_L) which comprises in sequence CDRL1, CDRL2 and CDRL3 from table 3; or

an anti-Nogo antibody or functional fragment thereof which comprises:

a) a heavy chain variable domain (V_H) which comprises in sequence CDRH1, CDRH2 and CDRH3 from table 6,

and /or

c) a light chain variable domain (V_L) which comprises in sequence CDRL1, CDRL2 and CDRL3 from table 5.

The anti-Nogo antibody or functional fragment thereof may preferably comprise a heavy chain variable region comprising one of the following amino acid sequences:-

QVQLQQPGTELVKPGASVKLSCKASGYTFTSYWMHWVKQRPGQGLEWIGNINPSNGGTN
YNEKFKSKATLTVDKSSSTAYMQLSSLTSEDSAVYYCELGQGYWGQGTTLTVSS
(SEQ ID NO: 37); or

EVQLVESGGGLVKPGGSLKLSCAASGFTFSCYAMSWVRQTPEKRLEWVASISDGGSYTY
YPDNVKGRFTISRDNAKNNLYLQMSHLKSEDTAMYYCAKELLFDYWGQGTTLTVSS
(SEQ ID NO:38); or

QVQLQQSGAELVKPGASVKISCKASGYAFSSYWMNWVKQRPGKGLEWIGQIYPGDGDTN
YNGKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYFCAVRFDYWGQGTTLTVSS
(SEQ ID NO:39).

Further, the anti-Nogo antibody or functional fragment thereof may preferably comprise a light chain variable region comprising one of the following amino acid sequences:-

DIVITQDELSNPVTSGESVSISCRSSKSLLYKDGKTYLNWFLQRPGQSPQLLIYLMSTR
ASGVSDRFSGSGSGTDFTLEISRVKAEDVGVYYCQQLVEYPLTFGAGTKLELK
(SEQ ID NO:40); or

DVVMTQTPLSLPVSLGDQASISC[RSSQSLVHSNGNTYLH]WYLQKPGQSPKLLIY[KVSNR]
[FS]GVPDRFSGSGSGTDFTLKISRVEAEDLGVYFC[SQSTHVPLT]FGAGTKLELK
(SEQ ID NO:41); or

DIVMTQAAPSVPVTPGESVSISC[RSSKSLLHSNGNTYLY]WFLQRPGQSPQLLIY[RMSNL]
[AS]GVPDRFSGSGSGTAFTLRISRVEAEDVGVYYC[MQHLEYPLT]FGAGTKLELK
(SEQ ID NO:42).

The boxed sequences in SEQ ID Nos 37 to 42 represent the CDR sequences according to Kabat et al *supra*.

In a preferred embodiment the anti-Nogo antibody comprises:
a) a heavy chain variable region of SEQ ID NO:37 together with a light chain variable region comprising the amino acid sequence of SEQ ID NO:40; or
b) a heavy chain variable region of SEQ ID NO:38 together with a light chain variable region comprising the amino acid sequence of SEQ ID NO:41; or
c) a heavy chain variable region of SEQ ID NO:39 together with a light chain variable region comprising the amino acid sequence of SEQ ID NO:42.

More preferably the anti-Nogo antibody, or functional fragment thereof, comprises either:
a heavy chain variable fragment comprising SEQ ID NO:37 and a constant part or fragment thereof of a human heavy chain; and
a light chain variable fragment comprising SEQ ID No:40 and a constant part or fragment thereof of a human light chain; or
a heavy chain variable fragment comprising SEQ ID NO:38 and a constant part or fragment thereof of a human heavy chain; and
a light chain variable fragment comprising SEQ ID No:41 and a constant part or fragment thereof of a human light chain; or
a heavy chain variable fragment comprising SEQ ID NO:39 and a constant part or fragment thereof of a human heavy chain; and
a light chain variable fragment comprising SEQ ID No:42 and a constant part or fragment thereof of a human light chain.

Still more preferably the anti-Nogo antibody is selected from 2A10/3, 2C4/1 or 15C3/3, preferably the humanised form thereof. Most preferably the anti-Nogo antibody is 2A10/3 or the humanised form thereof.

Anti-Nogo antibody 2A10/3 comprises a heavy chain variable region having the amino acid sequence of SEQ ID NO:37 and a light chain variable region having the amino acid sequence of SEQ ID NO:40.

Anti-Nogo antibody 2C4/1 comprises a heavy chain variable region having the amino acid sequence of SEQ ID NO:38 and a light chain variable region having the amino acid sequence of SEQ ID NO:41.

Anti-Nogo antibody 15C3/3 comprises a heavy chain variable region having the amino acid sequence of SEQ ID NO:39 and a light chain variable region having the amino acid sequence of SEQ ID NO:42.

The anti-Nogo antibody may be prepared using standard methods. In particular the Nogo antibody may be prepared using polynucleotides as described below. For example preferred polynucleotides encoding CDRH1, CDRH2, CDRH3, CDRL1, CDRL2 and CDRL3, as disclosed in tables 1 to 6, are shown below in tables 7 to 12.

Table 7: Antibody 2A10/3 light chain CDRs

CDR	
L1	AGGTCTAGTAAGAGTCTCCTATATAAGGATG GGAAGACATACTTGAAT (SEQ ID NO:19)
L2	TTGATGTCCACCCGTGCATCA (SEQ ID NO:20)
L3	CAACAACTTGTAGAGTATCCGCTCACG (SEQ ID NO:21)

Table 8: Antibody 2A10/3 heavy chain CDRs

CDR	
H1	AGCTACTGGATGCAC (SEQ ID NO:22)

H2	AATATTAATCCTAGCAATGGTGGTACTAACTACAAT GAGAAGTTCAAGAGC (SEQ ID NO:23)
H3	GGACAGGGCTAC (SEQ ID NO:24)

Table 9: Antibody 2C4/1 light chain CDRs

CDR	
L1	AGATCTAGTCAGAGCCTTGTACACAGTAATG GAAACACCTATTTACAT (SEQ ID NO:25)
L2	AAAGTTTCCAACCGATTTTCT (SEQ ID NO:26)
L3	TCTCAGAGTACACATGTTCCG CTCACG (SEQ ID NO:27)

Table 10: Antibody 2C4/1 heavy chain CDRs

CDR	
H1	TTCAGTTGCTATGCCATGTCT (SEQ ID NO:28)
H2	TCCATTAGTGATGGTGGTAGTTACACCTACTATCCA GACAATGTAAAGGGC (SEQ ID NO:29)
H3	GAACTACTTTTTGACTAC (SEQ ID NO:30)

Table 11: Antibody 15C3/3 light chain CDRs

CDR	
L1	AGGTCTAGTAAGAGTCTCCTGCATAGTAATGGCAA CACTTACTTGTAT (SEQ ID NO:31)
L2	CGGATGTCCAACCTTGCCTCA (SEQ ID NO:32)
L3	ATGCAACATCTAGAATATCCGCTCACG (SEQ ID NO:33)

Table 12: Antibody 15C3/3 heavy chain CDRs

CDR	
H1	AGCTACTGGATGAAC (SEQ ID NO:34)
H2	CAGATTTATCCTGGAGATGGTGATACTAACTACAAC GGAAAGTTCAAGGGC (SEQ ID NO:35)
H3	CGCTTTGACTAT (SEQ ID NO:36)

Further, preferred polynucleotides encoding the heavy chain variable region comprising the amino acid sequences of SEQ ID NOs 37 to 39 and light chain variable regions comprising the amino acid sequences of SEQ ID NOs 40 to 42 are shown below.

A preferred polynucleotide sequence encoding the amino acid sequence of SEQ ID NO:37 is

CCAGGTCCAACTGCAGCAGCCTGGGACTGAACTGGTGAAGCCTGGGGCTTCAGTGAAGC
TGTCCTGCAAGGCTTCTGGCTACACCTTCACCAGCTACTGGATGCACTGGGTGAAGCAG
AGGCCTGGACAAGGCCTTGAGTGGATTGGAAATATTAATCCTAGCAATGGTGGTACTAA
CTACAATGAGAAGTTCAAGAGCAAGGCCACACTGACTGTAGACAAATCCTCCAGCACAG
CCTACATGCAGCTCAGCAGCCTGACATCTGAGGACTCTGCGGTCTATTATTGTGAACTG
GGACAGGGCTACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA
(SEQ ID NO:43)

A preferred polynucleotide sequence encoding the amino acid sequence of SEQ ID NO:38 is:

GAAGTGCAGCTGGTGGAGTCTGGGGGAGGCTTAGTGAAGCCTGGAGGGTC
CCTGAAACTCTCCTGTGCAGCCTCTGGATTCACTTTCAGTTGCTATGCCA
TGTCTTGGGTTCGCCAGACTCCGGAAAAGAGGCTGGAGTGGGTCGCATCC
ATTAGTGATGGTGGTAGTTACACCTACTATCCAGACAATGTAAAGGGCCG
ATTCACCATCTCCAGAGACAATGCCAAGAACAACCTGTACCTGCAAATGA
GCCATCTGAAGTCTGAGGACACAGCCATGTATTACTGTGCAAAGGAACTA
CTTTTTGACTACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA
(SEQ ID NO:44)

A preferred polynucleotide sequence encoding the amino acid sequence of SEQ ID NO:39 is:

CAGGTTCAGCTGCAGCAGTCTGGGGCTGAGCTGGTGAAGCCTGGGGCCTC
AGTGAAGATTTCCTGCAAAGCTTCTGGCTACGCATTCAGTAGCTACTGGA
TGAACTGGGTGAAGCAGAGGCCTGGAAAGGGTCTTGAGTGGATTGGACAG

ATTTATCCTGGAGATGGTGATACTAACTACAACGGAAAGTTCAAGGGCAA
GGCCACACTGACTGCAGACAAATCCTCCAGCACAGCCTACATGCAGCTCA
GCAGCCTGACCTCTGAGGACTCTGCGGTCTATTTCTGTGCAGTACGCTTT
GACTATTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA
(SEQ ID NO:45)

A preferred polynucleotide sequence encoding the amino acid sequence of SEQ ID NO:40 is:

GATATTGTGATAACCCAGGATGAACTCTCCAATCCTGTCACTTCTGGAGA
ATCAGTTTCCATCTCCTGCAGGTCTAGTAAGAGTCTCCTATATAAGGATG
GGAAGACATACTTGAATTGGTTTCTGCAGAGACCAGGACAATCTCCTCAG
CTCCTGATCTATTTGATGTCCACCCGTGCATCAGGAGTCTCAGACCGGTT
TAGTGGCAGTGGGTCAGGAACAGATTTCACCCTGGAAATCAGTAGAGTGA
AGGCTGAGGATGTGGGTGTGTATTACTGTCAACAACTTGTAGAGTATCCG
CTCACGTTCGGTGCTGGGACCAAGCTGGAGCTGAAA
(SEQ ID NO:46)

A preferred polynucleotide sequence encoding the amino acid sequence of SEQ ID NO:41 is:

GATGTTGTGATGACCCAAACTCCACTCTCCCTGCCTGTCAGTCTTGGAGA
TCAAGCCTCCATCTCTTGCAGATCTAGTCAGAGCCTTGTACACAGTAATG
GAAACACCTATTTACATTGGTACCTGCAGAAGCCAGGCCAGTCTCCAAAG
CTCCTGATCTACAAAGTTTCCAACCGATTTTCTGGGGTCCCAGACAGGTT
CAGTGGCAGTGGATCAGGGACAGATTTCACACTCAAGATCAGCAGAGTGG
AGGCTGAGGATCTGGGAGTTTATTTCTGCTCTCAGAGTACACATGTTCCG
CTCACGTTCGGTGCTGGGACCAAGCTGGAGCTGAAA
(SEQ ID NO:47)

A preferred polynucleotide sequence encoding the amino acid sequence of SEQ ID NO:42 is:

GATATTGTGATGACTCAGGCTGCACCCTCTGTACCTGTCACTCCTGGAGA
GTCAGTATCCATCTCCTGCAGGTCTAGTAAGAGTCTCCTGCATAGTAATG
GCAACACTTACTTGTATTGGTTCCTGCAGAGGCCAGGCCAGTCTCCTCAG
CTCCTGATATATCGGATGTCCAACCTTGCCTCAGGAGTCCCAGACAGGTT
CAGTGGCAGTGGGTCAGGAACTGCTTTCACACTGAGAATCAGTAGAGTGG
AGGCTGAGGATGTGGGTGTTTATTACTGTATGCAACATCTAGAATATCCG
CTCACGTTCGGTGCTGGGACCAAGCTGGAGCTGAAA

(SEQ ID NO:48)

The method of the invention may be carried out *in-vitro* or *in-vivo*. When carried out *in-vitro* the method may be a model for amyloid deposition, APP processing, or BACE activity or it may provide a means to screen for compounds useful in the treatment of neurolgical diseases such as Alzheimer's disease.

When carried out *in-vivo* the method of the invention may be a method of prophylaxis or treatment of neurological diseases/disorders including, but not limited to, acute diseases such as stroke, traumatic brain injury and spinal cord injury as well as chronic diseases including Alzheimer's disease, fronto-temporal dementias (tauopathies), peripheral neuropathy, Parkinson's disease, Huntington's disease, Creutzfeldt-Jakob Disease (CJD), amyotropic lateral sclerosis (ALS), multiple sclerosis and inclusion body myositis.

In a preferred embodiment the method is a method of prophylaxis or treatment of neurological disease that is involving amyloidosis. In a more preferred embodiment the amyloidosis is precipitated by an amyloidogenic peptide derived from APP, preferably Aβ; that is Aβ40, Aβ42 or both Aβ40 and Aβ42. Still more preferably the invention relates to a method of prophylaxis or treatment of Alzheimer's disease, fronto-temporal dementias (tauopathies), peripheral neuropathy, Parkinson's disease, Huntington's disease, Creutzfeldt-Jakob Disease (CJD), multiple sclerosis, amyotropic lateral sclerosis (ALS) or inclusion body myositis. Most preferably the invention relates to a method of prophylaxis or treatment of Alzheimer's disease.

The method of prophylaxis or treatment comprises the use of a therapeutic agent or pharmaceutical composition in the treatment of the disease as described hereinbelow.

In another aspect the invention provides the use of a Nogo antagonist in the manufacture of a medicament for the prophylaxis or treatment of neurological diseases/disorders including, but not limited to, acute diseases such as stroke, traumatic brain injury and spinal cord injury as well as chronic diseases including Alzheimer's disease, fronto-temporal dementias (tauopathies), peripheral neuropathy, Parkinson's disease, Huntington's disease, Creutzfeldt-Jakob

Disease (CJD), amyotropic lateral sclerosis (ALS), multiple sclerosis and inclusion body myositis.

In a preferred embodiment the invention provides the use of Nogo antagonist for the manufacture of a medicament for the prophylaxis or treatment of a neurological disease that is involving amyloidosis. In a more preferred embodiment the amyloidosis is precipitated by an amyloidogenic peptide derived from APP, preferably Aβ; that is Aβ40, Aβ42 or both Aβ40 and Aβ42. Still more preferably the invention relates to the use of a Nogo antagonist for the manufacture of a medicament for the prophylaxis or treatment of Alzheimer's disease, fronto-temporal dementias (tauopathies), peripheral neuropathy, Parkinson's disease, Huntington's disease, Creutzfeldt-Jakob Disease (CJD), multiple sclerosis, amyotropic lateral sclerosis (ALS) or inclusion body myositis. Most preferably the invention relates to the use of a Nogo antagonist for the manufacture of a medicament for the prophylaxis or treatment of Alzheimer's disease.

The Nogo antagonist for use in the method of the invention, including the method of treatment or the use for the manufacture of a medicament as hereinabove defined, is preferably a NogoA antagonist, most preferably a monoclonal antibody.

Preferably the monoclonal antibody is a function-blocking anti-NogoA antibody as described hereinabove. An example of such a preferred function-blocking anti-NogoA antibody is 2A10/3 as described hereinabove, or most preferably a humanised version thereof. Other examples are 2C4/1 and 15C3/3 or humanised versions thereof.

The anti-NogoA antibody may also be an antibody which binds to the same epitope on the NogoA polypeptide as an antibody having the CDRs described above. Preferably the epitope comprises the region 586 to 785 (NogoA amino acid numbering). More preferably the epitope is comprised within the region 586 to 685 or 686 to 785.

The Nogo antagonist may be administered as a prophylactic or post injury, or as otherwise needed. The dose and duration of treatment relates to the relative duration of the molecules of the present invention in the human

circulation, and can be adjusted by one of skill in the art depending upon the condition being treated and the general health of the patient.

The mode of administration of the Nogo antagonist may be any suitable route which delivers the agent to the host. The Nogo antagonists and pharmaceutical compositions thereof are particularly useful for parenteral administration, i.e., subcutaneously, intrathecally, intraperitoneally, intramuscularly, intravenously, or intranasally.

Nogo antagonists may be prepared as pharmaceutical compositions containing an effective amount of the Nogo antagonist as an active ingredient in a pharmaceutically acceptable carrier. In the prophylactic agent of the invention, an aqueous suspension or solution containing the antagonist, preferably buffered at physiological pH, in a form ready for injection is preferred. The compositions for parenteral administration will commonly comprise a solution of the antagonist or a cocktail thereof dissolved in an pharmaceutically acceptable carrier, preferably an aqueous carrier. A variety of aqueous carriers may be employed, e.g., 0.9% saline, 0.3% glycine, and the like. These solutions are sterile and generally free of particulate matter. These solutions may be sterilized by conventional, well known sterilization techniques (e.g., filtration). The compositions may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, etc. The concentration of the antagonist in such pharmaceutical formulation can vary widely, i.e., from less than about 0.5%, usually at or at least about 1% to as much as 15 or 20% by weight and will be selected primarily based on fluid volumes, viscosities, etc., according to the particular mode of administration selected.

Thus, a pharmaceutical composition for intramuscular injection could be prepared to contain 1 mL sterile buffered water, and between about 1 ng to about 100 mg, e.g. about 50 ng to about 30 mg or more preferably, about 5 mg to about 25 mg, of a Nogo antagonist. Similarly, a pharmaceutical composition of the invention for intravenous infusion could be made up to contain about 250 ml of sterile Ringer's solution, and about 1 to about 30 and preferably 5 mg to about 25 mg of an antagonist. Actual methods for preparing parenterally administrable compositions are well known or will be apparent to those skilled in the art and are

described in more detail in, for example, Remington's Pharmaceutical Science, 15th ed., Mack Publishing Company, Easton, Pennsylvania.

It is preferred that the Nogo antagonist, when in a pharmaceutical preparation, be present in unit dose forms. The appropriate therapeutically effective dose can be determined readily by those of skill in the art. To effectively treat neurological diseases in a human, one dose of up to 700 mg per 70 kg body weight of an antagonist should be administered parenterally, preferably *i.v.* or i.m. (intramuscularly). Such dose may, if necessary, be repeated at appropriate time intervals selected as appropriate by a physician.

The Nogo antagonists described herein can be lyophilized for storage and reconstituted in a suitable carrier prior to use. Where the Nogo antagonist is an antibody this technique has been shown to be effective with conventional immunoglobulins and art-known lyophilization and reconstitution techniques can be employed.

The following examples illustrate the invention.

Examples

Example 1 – Transfection of Nogo A cDNA into SHSY5Y-APP cells

The day before transfection the SHSY5Y-APPwt cells were trypsinised, counted and replated at the required cell density usually 0.2-1x10^6 cells per well of either a 6 or a 96-well plate (Nunc).

The Nogo expression construct (FLAG-tagged NogoA cDNA in pCDNA3 (Invitrogen); MYC-tagged Nogo-B and Nogo-C in pCDNA3.1A) was complexed with Plus™ reagent by diluting the DNA into serum free medium (OptiMEM-1), adding Plus™ reagent, mixing and incubating at room temperature for 15 min. (6ul Plus reagent into a total volume of 100ul with 2ug DNA and OptiMEM-1 per well)

LipofectAMINE™ reagent was diluted into serum free medium (Optimem-I) in a second tube and mixed (4ul Lipofectamine in 100ul volume per well).

Pre-complexed DNA (from above) was combined with the diluted lipofectAMINE, mixed and incubated at room temp for 15 min.

Meanwhile the cells were washed with serum free medium (OptiMEM-I) and then fresh serum free medium was added to the cells (800ul per well).

The DNA-Plus-LipofectAMINE reagent complexes were then added to the cells (200ul), mixed gently and the cells incubated at 37oC for 5hr in 5% CO2.
After 5 hours 1ml serum containing growth medium was added to the cells and the cells incubated overnight or 2hours.
After 14 hours (or 2 hours) all medium was removed and replaced with 1ml (OptiMEM-1) per well.
After 48 hours medium conditioning the medium was collected and assayed for amyloid content as described in example 2.

Example 2 – Detection of Aβ peptide by BioVeris (BV Technology) ELISA
SHSY5Y cells overexpressing the human APPwt or Amyloid Precursor Protein Swedish variant sequence (APPswe) were seeded in 96 well Nunc plates at a density of $1x10^5$ cells/well.
After 24 hours the reagents (eg. antibody, peptides, compounds etc) for testing were added to the cells in a final volume of 120µl and cells incubated for 24hr.
The medium was removed from cells and 50ul was assayed for Aβ x-40 and 50µl for Aβ x-42 in an overnight BIOVERIS immunoassay employing Aβ C-terminal specific antibodies. Briefly, Aβ peptides were captured using biotinylated 6E10 (Signet Labs). BV-tagged labelled Aβ C-terminal specific antibodies were used to detect the Aβ x-40 and Aβ x-42 species. Antibody- Aβ complexes were captured with streptavidin coated dynabeads and assayed in an BIOVERIS M-Series M384 analyser.
The viability of the cells was checked using MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue) reagent. Briefly, MTT reagent (5mg/ml in phosphate buffered saline) was diluted 1:10 in culture medium and 100µl added to each well. Following incubation at 37°C for 4hr, 100µl solubilising solution (20% SDS/50% Dimethyl Formamide) was added. Absorbance of plates was read using a Delfia Wallac plate reader at 590nM.

The results are shown in figures 1 to 14.

SHSY5Y-APPwt cells express wild-type APP. SHSY5Y-APPswe cells express the Swedish mutation form of APP.

Example 3 - The effect of NogoA expression on levels of secreted Aβ 40 and Aβ 42 peptide

When an expression construct expressing NogoA is introduced into either SHSY5Y-APPwt cells or SHSY5Y-APPswe cells the levels of Aβ 40 and Aβ 42 are seen to significantly increase suggesting that NogoA is in some way modulating, directly or indirectly, the proteolyic processing of APP and/ or degradation of Aβ peptides. The fact that the product of this altered APP processing is both the peptides Aβ40 and Aβ42 could suggest that the effect of Nogo could be at the level of modulating β-secretase activity, or altering peptide secretion or affecting stability or via currently less well characterised mechanisms.

Figure 1 shows the increase in the level of secreted Aβ40 when NogoA is expessed from an expression vector. The two left-had bars are the controls (vector alone and vector carrying a control protein, green fluorescent protein (GFP)), showing the backgound levels of Aβ40 production in this cell-line. The remaining bars shows that significantly increased level of Aβ40 peptide are detected when NogoA, fused to a FLAG peptide, is expressed in the cells and also that a similar elevation, albeit less marked, when Nogo-B, fused to myc, is expressed.

The same experiment was repeated using an ELISA specific for Aβ42. The results showed a similar elevation in levels of Aβ 42 peptide secretion as that seen in the earlier experiment using Aβ 40 ELISA. Again Nogo-B also showed increased secretion of Aβ 42 peptide and again the increase was less marked than with NogoA. The results are shown in figure 2.

Repeat experiments comparing levels of secreted peptide from cells transfected with NogoA compared to vector pCDNA3 alone, are shown in figures 3 (for Aβ 40) and 4 (for Aβ 42).

Figure 5 shows anormalised and averaged summary of data from three (Nogo-C) to five (vector, GFP, NogoA and –B) independent experiments to assess the reproducibility of the effect of Nogo expression on Aβ 40 and Aβ 42 peptide production.

Example 4 – Preparation of an anti-Nogo antibodies.

Preparation and selection of the hybridomas

Anti-Nogo monoclonal antibodies are produced by hybridoma cells, the result of the fusion of mouse myeloma cells with B lymphocytes from mice immunised with the target antigen. The hybridoma cell is immortalised by the myeloma fusion partner while the capacity to produce antibodies is provided by the B lymphocyte. Each hybridoma cell makes only one individual antibody with unique specificity hence the term monoclonal.

SJL mice were immunised with 10ug total protein (1:1, human NogoA splice (amino acids 186-1004) and rat NogoA splice (amino acids 173-975), produced as GST-fusion proteins in E coli BL21) using both CFA and RIBI adjuvants subcutaneously. The mice were then boosted with 5ug of the same proteins using RIBI adjuvant after 4 and 8 days. After a further 3 days, immune cells were harvested from the locally draining lymph nodes and fused with mouse myeloma cells using PEG1500 to generate hybridomas. Individual hybridoma cell lines were cloned by two rounds of limiting dilution.

Initial hybridoma antibody selection was on the basis of direct binding to the Nogo protein(s) on microtitre plates. Subsequently ca. 60 hybridomas were selected based on the ability of soluble protein (consisting of human NogoA sequence cleaved from the GST moiety using PreScission protease) to compete for this binding activity in ELISA assays.

Cloning of the variable regions

Total RNA was extracted from the selected 2A10/3, 2C4/1 and 15C3/3 hybridoma cells followed by reverse transcription and polymerase chain reaction (RT-PCR) to extract heavy and light variable-domain cDNA sequence. The forward primer for RT-PCR was a mixture of degenerate primers specific for murine immunoglobulin gene leader-sequences and the reverse primer was an isotype-specific antibody directed to the constant regions. PCR primers were designed to carry 5' restriction enzyme recognition sites to enable cloning into pUC19 for DNA sequencing.

RNA extraction

Total RNA was extracted from pellets of 10^6 cells of each hybridoma clone using the SV Total RNA Isolation System from Promega according to manufacturer's instructions.

Reverse transcription

RNA was reverse transcribed to produce cDNA of the variable heavy and light domains using forward primers specific for the murine leader sequences and reverse primers to murine IgGκ constant regions. The IgGγ1 reverse primer was used for hybridomas 2C4/1 and 15C3/3; and the IgGγ2b for 2A10/3. Forward primers carry a SalI restriction enzyme recognition site at the 5' end, with four extra nucleotides added 5'to this for efficient restriction digestion. These primers were adapted from Jones ST and Bendig MM 1991 (Biotechnology 9, 88-89). Reverse primers carry a XmaI restriction enzyme recognition site plus and extra four nucleotides at the 5' ends.

Primers:

Murine V_H leader sequence forward primers:

AG77: 5'-ACT AGT CGA CAT GAA ATG CAG CTG GGT CAT STT CTT C-3'

AG78: 5'-ACT AGT CGA CAT GGG ATG GAG CTR TAT CAT SYT CTT-3'

AG79: 5'-ACT AGT CGA CAT GAA GWT GTG GTT AAA CTG GGT TTT T-3'

AG80: 5'-ACT AGT CGA CAT GRA CTT TGG GYT CAG CTT GRT TT-3'

AG81: 5'-ACT AGT CGA CAT GGA CTC CAG GCT CAA TTT AGT TTT CCT T-3'

AG82: 5'-ACT AGT CGA CAT GGC TGT CYT RGS GCT RCT CTT CTG C-3'

AG83: 5'-ACT AGT CGA CAT GGR ATG GAG CKG GRT CTT TMT CTT-3'

AG84: 5'-ACT AGT CGA CAT GAG AGT GCT GAT TCT TTT GTG-3'

AG85: 5'-ACT AGT CGA CAT GGM TTG GGT GTG GAM CTT GCT ATT CCT G-3'

AG86: 5'-ACT AGT CGA CAT GGG CAG ACT TAC ATT CTC ATT CCT G-3'

AG87: 5'-ACT AGT CGA CAT GGA TTT TGG GCT GAT TTT TTT TAT TG-3'

AG89: 5'-ACT AGT CGA CAT GAT GGT GTT AAG TCT TCT GTA CCT G-3'

Murine V_L leader sequence forward primers:

AG90: 5'-ACT AGT CGA CAT GAA GTT GCC TGT TAG GCT GTT GGT GCT G-3'

AG91: 5'-ACT AGT CGA CAT GGA GWC AGA CAC ACT CCT GYT ATG GGT-3'

AG92: 5'-ACT AGT CGA CAT GAG TGT GCT CAC TCA GGT CCT GGC GTT G-3'

AG93: 5'-ACT AGT CGA CAT GAG GRC CCC TGC TCA GWT TYT TGG MWT CTT G-3'

AG94: 5'-ACT AGT CGA CAT GGA TTT WCA GGT GCA GAT TWT CAG CTT C-3'

AG95: 5'-ACT AGT CGA CAT GAG GTK CYY TGY TSA GYT YCT GRG G-3'

AG96: 5'-ACT AGT CGA CAT GGG CWT CAA GAT GGA GTC ACA KWY YCW GG-3'

AG97: 5'-ACT AGT CGA CAT GTG GGG AYC TKT TTY CMM TTT TTC AAT TG-3'

AG98: 5'-ACT AGT CGA CAT GGT RTC CWC ASC TCA GTT CCT TG-3'

AG99: 5'-ACT AGT CGA CAT GTA TAT ATG TTT GTT GTC TAT TTC T-3'

AG100: 5'-ACT AGT CGA CAT GGA AGC CCC AGC TCA GCT TCT CTT CC-3'

MKV12: 5'-ACT AGT CGA CAT GAA GTT TCC TTC TCA ACT TCT GCT C-3'

Murine γ1 constant region reverse primer:

AG102: 5'-GGA TCC CGG GCC AGT GGA TAG ACA GAT G-3'

Murine γ2b constant region reverse primer:

AG104: 5'-GGA TCC CGG GAG TGG ATA GAC TGA TGG-3'

Murine κ constant region reverse primer:

AG101: 5'-GGA TCC CGG GTG GAT GGT GGG AAG ATG-3'

Pools of murine V_H or V_L leader sequence forward primers were prepared at 50μM. Solutions of the murine γ or κ constant region reverse primers were also prepared at 50μM.

RT-PCR

Reverse transcription of the RNA encoding the variable heavy and light regions was carried out in duplicate using the Access RT-PCR System from Promega according to manufacturer's instructions. Approximately 200ng RNA was included in a 50 μl reaction containing RT-PCR buffer supplied, 0.2 mM dNTPs, 1uM of each primer set, 1uM MgS04 and 5U each of AMV Reverse transcriptase and Tfl DNA polymerase.

RT-PCR cycle:
1- 48°C for 45min
2- 94°C for 2min
3- 94°C for 30sec
4- 50°C for 1min
5- 68°C for 2min
6- 68°C for 7min
steps 3 to 5: repeat 30 times.

pUC19 cloning

The variable region RT-PCR products were purified using a Qiagen MinElute Qiagen PCR Purifcation kit according to their instructions and digested sequentially with Xmal and Sall from New England Biolabs according to manufacturer's instructions. They were then loaded on a preparative 1% agarose gel containing 0.5% ethidium bromide and run in TAE buffer at 50mA for 1hour and the V region bands excised under ultra-violet light. The DNA fragments were purified from the gel using the MinElute Gel extraction kit from Qiagen according to manufacturer's instructions. pUC19 vector arms were prepared by digesting pUC19 with Sall and Xmal, then purified using the MinElute Reaction Clean up kit from Qiagen and dephosphorylated using Shrimp alkaline phosphatase (USB) according to the manufacturer's instructions. The concentration of the vector arms and the V-region fragments was estimated from an analytical 1% agarose/ethidium bromide gel, mixed in a molar ratio of 1:2 and ligated using Promega's Quick Ligation kit acording to the manufacturer's instructions. Ligated plasmids were transformed into DH5a cells (Invitrogen) according manufacturer's instructions. Colonies which grew on L-agar plates containing 100ug/ml ampicillin were selected for DNA sequence analysis.

Variable region sequencing

Colonies were cultured overnight at 37°C in 5ml LB medium supplemented with 100µg/ml ampicillin amd plasmid DNA was extracted and purified using the Qiagen QIAprep Spin Miniprep kit according to manufacturer's instructions. The V_H and V_L regions were DNA sequenced using standard M13 forward and reverse primers.

The results of the sequencing determination are shown as SEQ ID NOs 43 to 48.

Heavy chain variable regions:

2A10/3

CCAGGTCCAACTGCAGCAGCCTGGGACTGAACTGGTGAAGCCTGGGGCTTCAGT
GAAGCTGTCCTGCAAGGCTTCTGGCTACACCTTCACCAGCTACTGGATGCACTG
GGTGAAGCAGAGGCCTGGACAAGGCCTTGAGTGGATTGGAAATATTAATCCTAG
CAATGGTGGTACTAACTACAATGAGAAGTTCAAGAGCAAGGCCACACTGACTGT
AGACAAATCCTCCAGCACAGCCTACATGCAGCTCAGCAGCCTGACATCTGAGGA
CTCTGCGGTCTATTATTGTGAACTGGGACAGGGCTACTGGGGCCAAGGCACCAC
TCTCACAGTCTCCTCA

(SEQ ID NO:43)

QVQLQQPGTELVKPGASVKLSCKASGYTFTSYWMHWVKQRPGQGLEWIGNINPS
NGGTNYNEKFKSKATLTVDKSSSTAYMQLSSLTSEDSAVYYCELGQGYWGQGTT
LTVSS
(SEQ ID NO: 37)

2C4/1

GAAGTGCAGCTGGTGGAGTCTGGGGGAGGCTTAGTGAAGCCTGGAGGGTC
CCTGAAACTCTCCTGTGCAGCCTCTGGATTCACTTTCAGTTGCTATGCCA
TGTCTTGGGTTCGCCAGACTCCGGAAAAGAGGCTGGAGTGGGTCGCATCC
ATTAGTGATGGTGGTAGTTACACCTACTATCCAGACAATGTAAAGGGCCG
ATTCACCATCTCCAGAGACAATGCCAAGAACAACCTGTACCTGCAAATGA
GCCATCTGAAGTCTGAGGACACAGCCATGTATTACTGTGCAAAGGAACTA
CTTTTTGACTACTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA
(SEQ ID NO:44)

EVQLVESGGGLVKPGGSLKLSCAASGFTFSCYAMSWVRQTPEKRLEWVASISDG
GSYTYYPDNVKGRFTISRDNAKNNLYLQMSHLKSEDTAMYYCAKELLFDYWGQG
TTLTVSS
(SEQ ID NO:38)

15C3/3

CAGGTTCAGCTGCAGCAGTCTGGGGCTGAGCTGGTGAAGCCTGGGGCCTC
AGTGAAGATTTCCTGCAAAGCTTCTGGCTACGCATTCAGTAGCTACTGGA
TGAACTGGGTGAAGCAGAGGCCTGGAAAGGGTCTTGAGTGGATTGGACAG
ATTTATCCTGGAGATGGTGATACTAACTACAACGGAAAGTTCAAGGGCAA
GGCCACACTGACTGCAGACAAATCCTCCAGCACAGCCTACATGCAGCTCA
GCAGCCTGACCTCTGAGGACTCTGCGGTCTATTTCTGTGCAGTACGCTTT
GACTATTGGGGCCAAGGCACCACTCTCACAGTCTCCTCA
(SEQ ID NO:45)

QVQLQQSGAELVKPGASVKISCKASGYAFSSYWMNWVKQRPGKGLEWIGQIYPG
DGDTNYNGKFKGKATLTADKSSSTAYMQLSSLTSEDSAVYFCAVRFDYWGQGTT
LTVSS
(SEQ ID NO:39)

Light chain variable regions:

2A10/3

GATATTGTGATAACCCAGGATGAACTCTCCAATCCTGTCACTTCTGGAGA

ATCAGTTTCCATCTCCTGCAGGTCTAGTAAGAGTCTCCTATATAAGGATG
GGAAGACATACTTGAATTGGTTTCTGCAGAGACCAGGACAATCTCCTCAG
CTCCTGATCTATTTGATGTCCACCCGTGCATCAGGAGTCTCAGACCGGTT
TAGTGGCAGTGGGTCAGGAACAGATTTCACCCTGGAAATCAGTAGAGTGA
AGGCTGAGGATGTGGGTGTGTATTACTGTCAACAACTTGTAGAGTATCCG
CTCACGTTCGGTGCTGGGACCAAGCTGGAGCTGAAA
(SEQ ID NO:46)

DIVITQDELSNPVTSGESVSISC[RSSKSLLYKDGKTYLN]WFLQRPGQSPQLLIY
[LMSTRAS]GVSDRFSGSGSGTDFTLEISRVKAEDVGVYYC[QQLVEYPLT]FGAGTK
LELK
(SEQ ID NO:40)

2C4/1

GATGTTGTGATGACCCAAACTCCACTCTCCCTGCCTGTCAGTCTTGGAGA
TCAAGCCTCCATCTCTTGCAGATCTAGTCAGAGCCTTGTACACAGTAATG
GAAACACCTATTTACATTGGTACCTGCAGAAGCCAGGCCAGTCTCCAAAG
CTCCTGATCTACAAAGTTTCCAACCGATTTTCTGGGGTCCCAGACAGGTT
CAGTGGCAGTGGATCAGGGACAGATTTCACACTCAAGATCAGCAGAGTGG
AGGCTGAGGATCTGGGAGTTTATTTCTGCTCTCAGAGTACACATGTTCCG
CTCACGTTCGGTGCTGGGACCAAGCTGGAGCTGAAA
(SEQ ID NO:47)

DVVMTQTPLSLPVSLGDQASISC[RSSQSLVHSNGNTYLH]WYLQKPGQSPKLLIY
[KVSNRFS]GVPDRFSGSGSGTDFTLKISRVEAEDLGVYFC[SQSTHVPLT]FGAGTK
LELK
(SEQ ID NO:41)

15C3/3

GATATTGTGATGACTCAGGCTGCACCCTCTGTACCTGTCACTCCTGGAGA
GTCAGTATCCATCTCCTGCAGGTCTAGTAAGAGTCTCCTGCATAGTAATG
GCAACACTTACTTGTATTGGTTCCTGCAGAGGCCAGGCCAGTCTCCTCAG
CTCCTGATATATCGGATGTCCAACCTTGCCTCAGGAGTCCCAGACAGGTT
CAGTGGCAGTGGGTCAGGAACTGCTTTCACACTGAGAATCAGTAGAGTGG
AGGCTGAGGATGTGGGTGTTTATTACTGTATGCAACATCTAGAATATCCG
CTCACGTTCGGTGCTGGGACCAAGCTGGAGCTGAAA
(SEQ ID NO:48)

DIVMTQAAPSVPVTPGESVSISCRSSKSLLHSNGNTYLYWFLQRPGQSPQLLIY
RMSNLASGVPDRFSGSGSGTAFTLRISRVEAEDVGVYYCMQHLEYPLTFGAGTK
LELK
(SEQ ID NO:42).

The DNA sequences for the light or heavy chain regions are given for the antibodies 2A10/3, 2C4/1 and 15C3/3. Below each DNA sequence is the predicted amino acid sequence encoded by that DNA and the CDRs are shown in boxes.

Recombinant anti-Nogo antibodies

Recombinant antibodies having murine 2a/k constant regions could be purified from cells transfected with plasmids comprising the light and heavy variable regions cloned onto mouse IgG2a/k constant region gene segments. The cloned murine V regions were amplified by PCR to introduce restriction sites required for cloning into mammalian expression vectors RId and RIn. Hind III and Spe I sites were designed in frame with the V_H domain to allow cloning into a modified RId vector containing the mouse γ2a constant region. Hind III and BsiW I sites were designed in frame the V_L domain and allow cloning into a modified RIn vector containing the mouse κ constant region.

PCR primers

2A10 V_H forward primer:

5'-
ACTCATAAGCTTGCCACCATGGGATGGAGCTGTATCATCCTCTTTTTGGTAG
-3'

V_H reverse primer:

5'-ACTATGACTAGTGTGCCTTGGCCCCAGTAG-3'

V_L forward primer:

5'- ACTCATAAGCTTGCCACCATGAGGTGCTCTCTTCAGTTTCTG -3'

V_L reverse primer:

5'- ACTATGCGTACGTTTCAGCTCCAGCTTGG -3'

PCR was performed using Hercules (Stratagene) according to the manufacturer's instructions in 50µl volume containing approx 10ng of the pUC19 miniprep containing the V-region, 2% DMSO, 400µM dNTPs, 1µM each primer and buffer supplied. PCR was carried out as follows 1-95 °C 2 mins, 2-95°C 1 min, 3-56 °C 1 min, 4-72°C 1 min. Steps 2-4 30 cycles.

Cloning into expression vectors

The PCR products were purified using the MinElute PCR Purification kit from Qiagen according to manufacturer's instructions. The V_H PCR product and Rld (IgG2a) mammalian expression vector were digested Hind III-Spe I. The V_L PCR product and Rln (k) mammalian expression vector were digested Hind III-BsiW I (NEB) according to manufacturer's instructions. Vectors were ligated to inserts in a 1:2 molar ratio using the Promega Quick Ligation kit. Ligation mixes were transfected into DH5a cells and colonies growing on ampicillin selection were grown up and sent for DNA sequence verification.

Sequencing of recombinant anti-Nogo antibody 2A10/3

The sequence of the 2A10 heavy chain between the HindIII and EcoRI cloning sites was determined to be:

AAGCTTGCCACCATGGGATGGAGCTGTATCATCCTCTTTTTGGTAGCAGC
AGCTACAGGTGTCCACTCCCAGGTCCAACTGCAGCAGCCTGGGACTGAAC
TGGTGAAGCCTGGGGCTTCAGTGAAGCTGTCCTGCAAGGCTTCTGGCTAC
ACCTTCACCAGCTACTGGATGCACTGGGTGAAGCAGAGGCCTGGACAAGG
CCTTGAGTGGATTGGAAATATTAATCCTAGCAATGGTGGTACTAACTACA
ATGAGAAGTTCAAGAGCAAGGCCACACTGACTGTAGACAAATCCTCCAGC
ACAGCCTACATGCAGCTCAGCAGCCTGACATCTGAGGACTCTGCGGTCTA
TTATTGTGAACTGGGACAGGGCTACTGGGGCCAAGGCACACTAGTCACCG
TCTCCTCAGCCAAAACAACAGCCCCATCGGTCTATCCACTGGCCCCTGTG
TGTGGAGATACAACTGGCTCCTCGGTGACTCTAGGATGCCTGGTCAAGGG
TTATTTCCCTGAGCCAGTGACCTTGACCTGGAACTCTGGATCCCTGTCCA
GTGGTGTGCACACCTTCCCAGCTGTCCTGCAGTCTGACCTCTACACCCTC
AGCAGCTCAGTGACTGTAACCTCGAGCACCTGGCCCAGCCAGTCCATCAC
CTGCAATGTGGCCCACCCGGCAAGCAGCACCAAGGTGGACAAGAAAATTG
AGCCCAGAGGGCCCACAATCAAGCCCTGTCCTCCATGCAAATGCCCAGCA
CCTAACCTCCTGGGTGGCCCATCCGTCTTCATCTTCCCTCCAAAGATCAA
GGATGTACTCATGATCTCCCTGAGCCCCATAGTCACATGTGTGGTGGTGG
ATGTGAGCGAGGATGACCCAGATGTCCAGATCAGCTGGTTTGTGAACAAC

GTGGAAGTACACACAGCTCAGACACAAACCCATAGAGAGGATTACAACAG
TACTCTCCGGGTGGTCAGTGCCCTCCCCATCCAGCACCAGGACTGGATGA
GTGGCAAGGAGTTCAAATGCAAGGTCAACAACAAAGACCTCCCAGCGCCC
ATCGAGAGAACCATCTCAAAACCCAAAGGGTCAGTAAGAGCTCCACAGGT
ATATGTCTTGCCTCCACCAGAAGAAGAGATGACTAAGAAACAGGTCACTC
TGACCTGCATGGTCACAGACTTCATGCCTGAAGACATTTACGTGGAGTGG
ACCAACAACGGGAAAACAGAGCTAAACTACAAGAACACTGAACCAGTCCT
GGACTCTGATGGTTCTTACTTCATGTACAGCAAGCTGAGAGTGGAAAAGA
AGAACTGGGTGGAAAGAAATAGCTACTCCTGTTCAGTGGTCCACGAGGGT
CTGCACAATCACCACACGACTAAGAGCTTCTCCCGGACTCCGGGTAAATG
AGAATTC

(SEQ ID NO:49)

The sequence of the 2A10 light chain between the HindIII and EcoRI cloning sites was determined to be:

AAGCTTGCCACCATGAGGTGCTCTCTTCAGTTTCTGGGGGTGCTTATGTT
CTGGATCTCTGGAGTCAGTGGGGATATTGTGATAACCCAGGATGAACTCT
CCAATCCTGTCACTTCTGGAGAATCAGTTTCCATCTCCTGCAGGTCTAGT
AAGAGTCTCCTATATAAGGATGGGAAGACATACTTGAATTGGTTTCTGCA
GAGACCAGGACAATCTCCTCAGCTCCTGATCTATTTGATGTCCACCCGTG
CATCAGGAGTCTCAGACCGGTTTAGTGGCAGTGGGTCAGGAACAGATTTC
ACCCTGGAAATCAGTAGAGTGAAGGCTGAGGATGTGGGTGTGTATTACTG
TCAACAACTTGTAGAGTATCCGCTCACGTTCGGTGCTGGGACCAAGCTGG
AGCTGAAACGTACGGATGCTGCACCGACTGTATCCATCTTCCCACCATCC
AGTGAGCAGTTAACATCTGGAGGTGCCTCAGTCGTGTGCTTCTTGAACAA
CTTCTACCCCAAAGACATCAATGTCAAGTGGAAGATTGATGGCAGTGAAC
GACAAAATGGCGTCCTGAACAGTTGGACTGATCAGGACAGCAAAGACAGC
ACCTACAGCATGAGCAGCACCCTCACGTTGACCAAGGACGAGTATGAACG
ACATAACAGCTATACCTGTGAGGCCACTCACAAGACATCAACTTCACCCA
TTGTCAAGAGCTTCAACAGGAATGAGTGTTAAGAATTC

(SEQ ID NO:50

Example 5 - anti-NogoA antibody inhibits Aβ 40 and Aβ 42 peptide secretion from SHSY5Y-APPwt and SHSY5Yswe cells

Figure 6 shows the dramatic reduction in levels of Aβ 40 and Aβ 42 peptide secreted from SHSY5Y-APPwt cells expressing endogenous NogoA when the anti-Nogo antibody 2A10 is introduced into the culture medium.

The effect is seen in a dose-dependent manner, at 30μg/ml reaching inhibition levels of almost 90%. There is no apparent difference in the effect between Aβ 40 and Aβ 42 peptides (white bars and black bars respectively in figure 6), in other words any effect of the anti-Nogo antibody on APP processing is not preferential for either the Aβ 40 or Aβ 42 peptides.

Figure 7 shows the same experiment as in figure 6 but with an unrelated IgG1 antibody. As can clearly be seen in the figure, an unrelated (non anti-Nogo A) monoclonal antibody has no inhibitory effect on the levels of Aβ 40 and Aβ 42 peptide secretion from the cells.

Figure 8 shows that the same unrelated IgG1 antibody similarly shows little or no inhibitory effect on the levels of Aβ 40 and Aβ 42 peptide secretion from SHSY5Y-APPswe cells expressing NogoA.

Similarly figure 9 shows the results of the same experiment as described above for figure 6 but using an anti-Nogo monoclonal antibody which binds to NogoA but is not a function-blocker (6D5). The non-function-blocking anti-NogoA monoclonal antibody has minimal effect (less than 10%) on the secretion of Aβ 40 and Aβ 42 peptides from SHSY5Y-APPwt cells expressing NogoA. This result suggests that the results shown in figure 6 are a result of the inhibition of Nogo functional activity by the anti-Nogo antibody.

Figure 10 shows the results of the same experiment as for figure 9, using the non-function blocking anti-Nogo monoclonal antibody (6D5) but with SHSY5Y-APPswe cells expressing endogenous NogoA. As before (figure 9) there is minimal effect on the levels of Aβ 40 and Aβ 42 being secreted by this cell line, being less than 10% inhibition.

Figure 11 shows the results of an experiment which extends the results of the experiment of figure 6. Figure 11 shows that the concentration-dependent effect of the inhibitory effect shown by the function-blocking antibody 2A10/3 continues at a higher concentration, a level of greater than 90% inhibition being achieved at an antibody concentration of 50μg/ml.

Figure 12 shows the results of an identical experiment to that of figure 11 except that SHSY5Yswe cells are used. The concentration-dependent inhibitory effect of the antibody 2A10/3 continues to be seen at the higher concentration of 50μg/ml.

Figure 13 shows the effect of a different function-blocking anti-NogoA antibody, 2C4, on the secretion of Aβ 40 and Aβ 42 peptides from SHSY5Y-APPwt cells. The results show a concentration-dependent inhibitory effect on the levels of Aβ 40 and Aβ 42 peptide secretion to a level of 36% at a concentration of 20μg/ml. Again the effect is seen on both Aβ 40 and Aβ 42 peptides.

Figure 14 compares the inhibitory effect of the anti-NogoA function-blocking antibodies 2A10, 2C4 and 15C3 (at the concentrations shown in the figure) with other control antibodies 10A4, IgG2b and 14D12. The figure shows that the effect on inhibition of Aβ 40 and Aβ 42 secretion is specific to the function-blocking anti-NogoA monoclonal antibodies.

Example 6 – Increased NogoA expression elevates Aβ levels in a dose-dependent manner

To investigate the effect of NogoA expression on Aβ levels, SHSY5Y-APPwt cells were transiently transfected with increasing amounts of C-terminal myc tagged NogoA cDNA. The total amount of cDNA (5ug) was kept constant for each transfection using pcDNA3.1myc cDNA. 48hr post-transfection, culture medium was removed and assayed for Aβ40. In addition, cells were harvested and lysed in 10mM Tris/HCl containing 1% Triton X-100 and Complete protease inhibitors (Roche). Cell lysates were resolved on 10% Novex Tris-glycine gels and subject to Western blot analysis with an anti-NogoA antibody. An increase in NogoA protein expression was observed with increasing NogoA cDNA concentrations (Fig 15). This was concomitant with a corresponding increase in Aβ40 levels in the medium from these cells. Thus, increased expression of NogoA causes a dose-dependent increase in Aβ40 levels.

In the figures:

Figure 1: Nogo A transfection leads to elevation of Aβ 40 peptide levels in SHSY5Y-APPwt cells

Figure 2: Nogo A transfection leads to elevation of Aβ 42 peptide levels in SHSY5Y-APPwt cells

Figure 3: Effect of Nogo A expression on Aβ 40 peptide levels

Figure 4: Effect of Nogo A expression on Aβ 42 peptide levels

Figure 5: Effect of Nogo A, Nogo-B and Nogo-C expression on Aβ 40 and Aβ 42

Figure 6. Anti-Nogo A antibody 2A10-BR inhibits Aβ secretion from SHSY5Y-APPwt cells

Figure 7. Effect of Chemicon IgG1 on Aβ secretion from SHSY5Y-APPwt cells

Figure 8. Effect of Chemicon IgG1 on Aβ secretion from SHSY5Y-APPswe cells

Figure 9. Effect of control anti-Nogo (non function-blocking) antibody 6D5 on Aβ secretion from SHSY5Y-APPwt cells

Figure 10. Effect of control anti-Nogo (non function-blocking) antibody 6D5 on Aβ secretion from SHSY5Y-APPswe cells

Figure 11. Function-blocking anti-Nogo A monoclonal antibody 2A10 inhibits Aβ secretion from SHSY5Y-APPwt cells

Figure 12. Function-blocking anti-Nogo A monoclonal antibody 2A10 inhibits Aβ secretion from SHSY5Y-APPswe cells

Figure 13. Function-blocking anti-Nogo A monoclonal antibody 2C4 inhibits Aβ secretion from SHSY5Y-APPwt cells

Figure 14. Effect of anti-Nogo A static culture antibody preparations and additional control antibodies on Aβ secretion from SHSY5Y-APPwt cells. 2A10, 2C4 and 15C3 are the static culture antibodies. All others are BR (Bioreactor) purified controls or commercially available controls.

Figure 15. Increased NogoA expression elevates Aβ levels in a dose-dependent manner. The Y axis of the graph is % increase of Aβ40. The X-axis shows the increasing concentration of myc-tagged NogoA cDNA. Above the graph is a gel showing the increased amount of NogoA protein expression as shown by western blotting using an anti-NogoA antibody.